AUTOXIDATION AND ANTIOXIDANTS

John M. Pezzuto
Eun Jung Park
University of Illinois at Chicago, Chicago, Illinois, U.S.A.

INTRODUCTION

By definition, oxygen is an absolute requirement for aerobic life, but it may also be viewed as toxic under certain conditions. A chemical reaction that usually takes place at ambient temperature between atmospheric oxygen and an organic compound is generally defined as autoxidation. The phenomena of autoxidation are commonly observed in everyday life. For example, the browning of fruit, deterioration of edible oils, and degeneration of old rubber bands are the results of autoxidation. In the human body, reactive oxygen species (ROS) produced during autoxidation processes are also commonly encountered. This is a normal part of human physiology, as long as defense systems are effective, but overproduction or failure to scavenge free radicals can result in toxic biological responses that can yield deterioration and degeneration of cells, tissues, or organisms.

There are numerous reports suggesting that free radicals are involved in various human disease states (Table 1). Pathological phenomena related to autoxidation are due to reactive oxygen species, and most age-related diseases can be explained through reactions of free radicals with biological substances. A notable case is cancer or, more specifically, carcinogenesis. Free radicals play a critical role in the initiation of carcinogenesis by damaging nucleic acids and producing a variety of lesions. For example, 8-hydroxyguanine, 5-hydroxymethyluracil, and thymine glycol are formed by the attack of hydroxyl free radicals on deoxyribonucleic acid (DNA), and these mutations may be viewed as a cause of carcinogenesis. Another free radical-related disease state is stroke. Damage to the brain due to central nervous system (CNS) ischemia is caused by injury resulting from the interruption of blood flow (i.e., lack of oxygenation) which is then followed by reoxygenation of the brain (ischemia/reperfusion). It appears that all or most aerobic tissue suffers damage once it undergoes an ischemia/reperfusion insult. The severity depends on many factors, one of which is the length of the ischemic period. Considerable evidence is now accumulating that injury occurs almost exclusively during the reperfusion phase, and that the injury is due to oxygen free radical-mediated oxidative stress. Furthermore, some neuronal pathologies, such as Alzheimer's disease, may relate to lipid peroxidation of cell membranes by free radicals.

Autoxidation phenomena could be completely prevented by total exclusion of oxygen or other oxidizing substances from a biological system. This is generally not practical, but changes in endogenous factors, such as addition of inhibitors, may decrease the reaction rate or prolong the induction period. However complete prevention of autoxidation is unlikely. Substances that can suppress autoxidation are termed inhibitors or antioxidants. Preventive inhibitors decrease the rate of autoxidation by suppressing the rate of initiation reactions. Antioxidants in the true sense are substances that can inhibit propagation steps; that is, they interrupt autoxidation chain reactions. Other types of inhibitors, for instance, antiozonants, are not biologically relevant but may be important in industry (e.g., protective coatings). Antioxidants are known to inhibit carcinogenesis or atherosclerosis. For example, vitamins and phenolic compounds can function as chemopreventive agents. The mechanism of action and the biological role of autoxidation and antioxidants are discussed in this article.

AUTOXIDATION

Autoxidation Reactions

The majority of compounds that are subject to autoxidation are unsaturated substances or highly condensed polymers, and the target of ROS is commonly the diene functionality—or double bonds. Autoxidation of unsaturated lipids is a good model to demonstrate this mechanism. The initial reaction between molecular oxygen and a polyunsaturated fatty acid (PUFA) occurs as $RH + O_2 \rightarrow ROOH$, which involves the movement of a double bond as well as the insertion of oxygen. In general, there is a "spin barrier" that prevents the direct addition of ground-state molecular oxygen in a single step to an organic compound. In the case of autoxidation, since direct addition is eliminated by the

Table 1 Structural and functional alterations of biological entities produced by radicals or ROS

Structure alterations	Functional alterations	Disease
Lipid peroxidation	Increased permeability of membrane	Cancer
Protein adducts	Impaired response of membrane to signals	Stroke
Hemoglobin adducts	Altered enzyme activity	Alzheimer's disease
	Reduced affinity of binding proteins	Rheumatoid arthritis
	Altered uptake of oxidized entities	Cataract

spin barrier, the alternatives are i) electron transfer (i.e., redox reactions) involving, for example, a transition metal ion, or ii) the participation of free radicals, whereby the addition of a radical to molecular oxygen can give rise to another radical (1).

The important features of autoxidation are autocatalytic and free radical chain reactions. The rate of oxidation is initially slow and increases as the reaction progresses. However, once autoxidation is initiated, the reaction continues until the reaction substrate or catalytic factor becomes extinct. In short, unsaturated lipids undergo three reaction phases: initiation, propagation, and termination. The participation of reactive oxygen radicals in autoxidation reactions is summarized in the following reaction steps.

$$\begin{aligned}
\text{Initiation:}\quad & X\cdot + RH \rightarrow R\cdot + XH \\
\text{Propagation:}\quad & R\cdot + O_2 \rightarrow RO_2\cdot \\
& RO_2\cdot + RH \rightarrow ROOH + R\cdot \\
\text{Termination:}\quad & RO_2\cdot + RO_2\cdot \rightarrow \text{Stable product} \\
& RO_2\cdot + R\cdot \rightarrow \text{Stable product} \\
& R\cdot + R\cdot \rightarrow \text{Stable product}
\end{aligned}$$

Initiation is perhaps the process most difficult to define. This is because of the very low concentration of radicals and the likelihood of there being more than one process, since a large number of different radicals can abstract hydrogen from RH to form $R\cdot$. For example, $X\cdot$ may be a transition-metal ion, a radical generated by photolysis or high-energy irradiation, a radical obtained by decomposition of a hydroperoxide (e.g., $RO\cdot$), or a radical formed from an exogenous initiator. The two propagation reactions form the basis of the chain-reaction process. The autoxidation reaction is generally assumed to be a very fast reaction with almost no activation energy. A major consequence of this phase is that the concentration of $R\cdot$ is much smaller than that of $RO_2\cdot$. Like initiation processes, termination reactions may also be divided into those involving organic free radicals exclusively, and those in which metal ions participate. Of the termination processes involving $R\cdot$ or $RO_2\cdot$, the biomolecular reaction

$$RO_2\cdot + RO_2\cdot \rightarrow \text{inert products}$$

has received the most attention since this is likely to be the most important termination reaction under physiological conditions.

Autoxidation of unsaturated lipids is affected by many factors, so that none can be considered exclusively prooxidative or antioxidative. The rate of autoxidation is increased with reactivity of the autoxidizing substrate, concentration of reactants (the number of active sites and the concentration of oxygen), modified physical factors (e.g., a rise in temperature or by irradiation), and especially with an increase in the rate of initiation reactions (1). The rate of chain initiation reactions is increased mainly by factors that increase free radical function in autoxidizing systems, for example, ultraviolet, X-Ray, or ionizing radiation. In various reactant systems, such as fats, oils, and biological membranes, heavy metals and their derivatives are important initiators of autoxidation reactions. The reaction rate, however, is suppressed by factors such as a reduction in the number of active sites, a decrease in the partial pressure of oxygen by a suitable selection of physical factors, and a decrease in the reaction temperature. The most important, however, is the reduction of initiation rate (i.e., the level of free radicals capable of chain initiation). Substances actively suppressing the concentration of the free radicals are antioxidants. Other substances are also very effective in suppressing autoxidation, particularly those eliminating free radical precursors (e.g., sulfur compounds that cause reduction of lipid hydroperoxide) (2). Substances deactivating ozone (antiozonants) or singlet oxygen, which protect against irradiation, or heavy metals (metal scavengers), also act as inhibitors of lipid autoxidation.

Lipid Peroxidation

Oxidative damage to membrane lipids (i.e., lipid peroxidation in biological systems) has been studied for many years. Lipid peroxidation is a primary event produced by oxidative stress or as a consequence of tissue damage, which can exacerbate tissue injury, due to the potential cytotoxicity and genotoxicity of the end products of lipid peroxidation. Membrane lipids with double bonds are most susceptible to oxidation. Lipid peroxidation can reduce membrane fluidity, leading to increased rigidity throughout the hydrophobic space of membranes, decreased permeability, osmotic fragility, and altered activity of certain membrane-bound enzymes and transport systems (3).

Membrane lipid peroxidation can change the activity of essential membrane proteins such as Na^+/K^+-ATPase. As a consequence, rates of ion pumping may be altered (4). Two well-characterized products of lipid peroxidation, malondialdehyde and 4-hydroxynonenal, have been shown to react with critical biomolecules that may have a key role in the development of certain pathological states. Microsomal lipid peroxidation forms mainly 4-hydroxy-2-nonenal with minor amounts of 4-hydroxy-2-octeal, 4-hydroxy-2-decenal, and 4-hydroxy-2-undecenal. α,β-Unsaturated aldehydes, such as 4-hydroxynonenal, are highly reactive electrophilic reagents that react easily with thiols by Michael addition to the CH=CH double bond (1, 5). Aldehyde adducts of lipid peroxidation have been shown to induce glutathione depletion in hepatocytes, and corresponding abnormal liver function (6, 7). Free hemoglobin, in the presence of xanthine/xanthine oxidase, will also promote the peroxidation of arachidonic acid and unsaturated fatty acids within normal cellular membranes. Furthermore, hemoglobin or red cell lysates cause brisk peroxidation of crude murine brain homogenates. This hemoglobin-driven peroxidation is blocked by an iron chelator (i.e., desferrioxamine) which indicates that iron released from heme is responsible (8).

Peroxides can play a physiological role in the cell but also mediate processes leading to heart disease and carcinogenesis (9–11). Fatty acid peroxidation may also be related to free radical-mediated metabolic activation of carcinogens or drugs, which lead to the initiation of carcinogenesis or cytotoxicity. Modification of membrane function as a consequence of lipid peroxidation includes uncoupling of oxidative phosphorylation in mitochondria, alteration of liver endoplasmic reticulum functions, and modification of the ionic permeability of phospholipid membranes. Radiation-catalyzed lipid peroxidation of erythrocyte membranes increases permeability to Na^+, K^+, and Ca^{++}. Ultimately, peroxidation leads to gross destruction of membranes, as demonstrated by release of lysosomal enzymes in irradiated lysosomes, hemolysis in irradiated erythrocytes, and release of various enzymes by disruption of the liver plasma membrane.

FREE RADICALS AND DEFENSE SYSTEMS

Free Radicals and Defense Systems

A free radical may be broadly defined as a molecule or ion containing an unpaired electron (Table 2). Although most radicals are reactive and undergo dimerization or other reactions in which the unpaired electron becomes paired some radicals such as nitroxide radicals ($R_2NO\cdot$) are relatively stable. A major source of radicals in biological systems is molecular oxygen. This very reactive molecule is essential to the life of higher organisms, but nevertheless can be considered dangerous in excess.

Superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are normal metabolites in mammalian cells produced during the biological reduction of oxygen. The occurrence of oxidative radical reactions in biological systems is usually associated with cellular electron transfer chains of the mitochondria and certain enzyme activities. Free radicals are further generated during the course of specialized physiological reactions, such as the release of $O_2^{\cdot -}$ and H_2O_2 by endothelium noninflammatory cells, which might serve as cell signals promoting growth responses. Furthermore, environmental factors as well as the metabolism of xenobiotics are significant sources of free radicals, although their actual contribution to the redox state of the cell is difficult to assess.

Although of modest chemical reactivity, $O_2^{\cdot -}$ and H_2O_2 contribute to the formation of more reactive species via various redox reactions. It is currently accepted that the reaction of both $O_2^{\cdot -}$ and H_2O_2 with suitable metal complexes yields $HO\cdot$. Fenton-type reactions requires both $O_2^{\cdot -}$ and H_2O_2 as precursors of $HO\cdot$. This proceeds via an intermediate catalyst, such as a transition metal chelate, which reacts to produce $HO\cdot$ (12). $HO\cdot$ may account for some aspects of mitochondrial damage, such as oxidative impairment of mitochondrial DNA. The requisite conditions for $HO\cdot$ formation by mitochondria are met during mitochondrial electron transfer: mitochondria are a major source of $HO\cdot$ generated by H_2O_2 cleavage.

$$\text{Haber} - \text{Weiss reactions:} \quad Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + H_2O_2$$

$$\text{Fenton reaction:} \quad Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + HO^-$$

Table 2 Principal reactive oxygen intermediates formed by cells

ROS	Function
Superoxide anion, $O_2^{\cdot -}$	One-electron reduction products of O_2, produced by phagocytes; formed in autoxidation reactions; generated by oxidases (heme protein).
Hydrogen peroxide, H_2O_2	Two-electron reduction product of O_2; formed from $O_2^{\cdot -}$ by dismutation or directly from O_2. Reactivity of $O_2^{\cdot -}$ and H_2O_2 amplified in the presence of heme protein.
Hydroxyl radical, HO·	Three-electron reduction product of $O_2^{\cdot}$ generated by Fenton reaction; transition metal (iron, copper)-catalyzed Haber–Weiss reaction; formed by decomposition of peroxynitrite produced by the reaction of $O_2^{\cdot -}$ with NO·.
Nitric oxide, NO·	Generated from arginine via NO synthase in an O_2-dependent reaction; in the absence of O_2, formed by Fe^{2+}-mediated nonenzymatic generation from tissue NO_2^-.

(Adapted in part from Ref. 15.)

Another type of one-electron transfer reaction that contributes to the formation of oxyradicals involves the quenching of carbon center radicals (R·) by molecular oxygen. This reaction leads to the formation of peroxyl radicals (R-OO·), which generally have quite different reactivities from those of the parent R· species. As a result of the reactivity of these species toward unsaturated fatty acids, the propagation steps of lipid peroxidation follow the initiation step. These propagation steps occur at membrane hydrophobic sites, and the length of the chain reaction is determined by the availability of reactants, PUFA and O_2, and of chain-breaking antioxidants such as α-tocopherol, carotenoids, and ubiquinone.

In cells, the first line of defense against these reactive species is represented by primary antioxidant defenses involving enzymes that specifically remove free radicals or oxidants, such as superoxide dismutase, glutathione peroxidase, and catalase. In general, aerobes do not express an excess of antioxidant defenses, although these systems are often inducible by elevated O_2 if sufficient time for adaptation is allowed. Moreover, endogenous antioxidants may not prevent damage by ROS at ambient O_2. Thus, animals rely on a second line of defense in the form of repair systems, the most important of which removes mutagenic lesions in DNA induced by ROS. Superimposed on such defenses are inducible proteins such as heme oxygenase-1. Heme oxygenases remove the prooxidant heme and produce the antioxidant bilirubin in the process (13). Additional protection is provided by dietary antioxidants. The physiological role of some of these is well established (e.g., vitamin E and ascorbate), whereas the role of others (e.g., flavonoids, carotenoids) is currently uncertain. However, dietary antioxidants appear to be important in delaying/preventing certain human diseases, especially cardiovascular disease and some types of cancer (14).

Antioxidants

Natural antioxidants such as nonenzymatic dietary components are not specific but can scavenge organic and inorganic radicals. These agents are found in numerous plant materials and commonly include an aromatic ring as part of their molecular structure. There are a variety of cyclic ring structures that are generally associated with one or more hydroxyl groups to provide a labile hydrogen and a basis for free radical formation. These antioxidants can be classified as water soluble or lipid-soluble, depending on whether they act primarily in the aqueous phase or in the lipophilic region of cell membranes. Hydrophilic antioxidants include ascorbic acid and urate. Ubiquinols, retinoids, carotenoids, flavonoids, and tocopherol are representative lipid-soluble antioxidants (Fig. 1). Plasma proteins, glutathione, and urate are endogenous, whereas ascorbic acid, carotenoids, retinoids, flavonoids, and tocopherols constitute some of the dietary antioxidants. These compounds possess the potential to scavenge and quench various radicals and ROS. Certain radical scavengers are not recyclable, however, others are recycled through the intervention of a series of enzyme systems or other nonenzymic antioxidant systems.

Based on safety and other pragmatic reasons, the number of active substances used as antioxidants is restricted to a few phenolic substances. The flavonoids are an unusually large group of naturally occurring phenolic compounds ubiquitously distributed in the plant kingdom (Fig. 2). These aromatic compounds are formed in plants from the aromatic amino acids, phenylalanine, tyrosine, and acetate units. Phenylalanine and tyrosine are converted to cinnamic acid and *p*-coumaric acid that condense with acetate units to form the cinnamoyl structure of the flavonoid (15). Flavonoids are generally

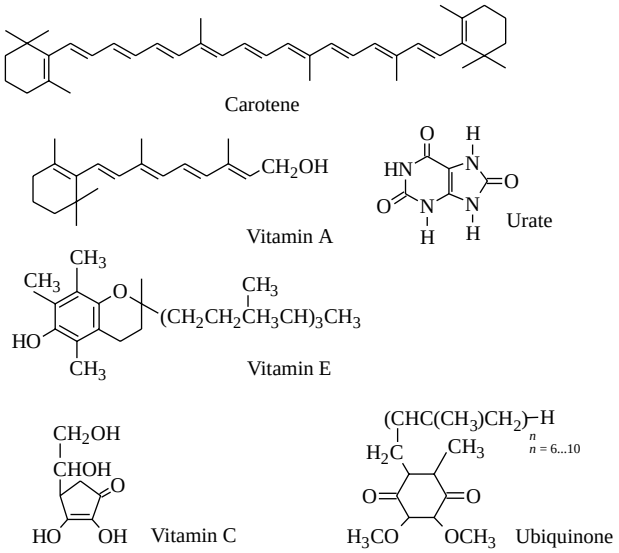


Fig. 1 Natural antioxidants.

known to be plant, flower, leaf, and fruit pigments. They are responsible for the brilliant shades of blue, scarlet, orange, etc., in flowers, fruits, and leaves. They are found in various fruits, vegetables, nuts, seeds, grains, spices, and herbs, as well as in beverages such as tea, cocoa, and wine. Dietary exposure to flavonoids is significant. The average diet in the United Kingdom and United States may contain up to 1 g of mixed flavonoids per day. Their dietary intake far exceeds that of vitamin E (a monophenolic antioxidant) and β-carotene (15).

Flavonoids act as potent metal chelators and free radical scavengers. They are powerful chain-breaking antioxidants (16). Moreover, flavonoids are known to possess vitamin C stabilizing and antioxidant-dependent vitamin C sparing activities. They are also known to increase the absorption of vitamin C. In addition, flavonoids are known to modify the activities of a host of enzyme systems including protein kinase C, protein tyrosine kinase and various other kinases, aldose reductase, myeloperoxidase, NADPH oxidase, xanthine oxidase, phospholipase, reverse transcriptases, ornithine decarboxylase, lipoxygenase, cyclooxygenase, and so on. Some of these enzyme systems are critically involved in immune function, carcinogenesis, cellular transformation, and tumor growth and metastasis. The physiologic and pathologic processes affected by flavonoids are diverse and numerous, and include secretion, mitogenesis, platelet aggregation and adhesion to endothelial surface, cell motility and malignant cell proliferation, cancer metastasis, and function/expression of adhesion molecules in various mammalian cell types. The antioxidant function and enzyme-modifying actions of flavonoids could account for many of their pharmacological activities (15).

Quercetin and other flavonoids are effective inhibitors of $O_2 \cdot$-production by cells. Quercetin is a potent inhibitor of human neutrophil degranulation and $O_2 \cdot$-production, and also inhibits the phosphorylation of neutrophil proteins accompanying neutrophil activation by phorbol myristate acetate (17). Quercetin can also suppress lipid peroxidation in several biological systems, such as mitochondria, microsomes, chloroplasts, and erythrocytes. Silymarin, a 3-OH flavone present in *Silybum marianum* (the European milk thistle), protects rat liver mitochondria and microsomes from lipid peroxide formation induced by Fe^{2+}-ascorbate and NADPH-Fe^{3+}-ADP systems (18). Soybean isoflavonoids have shown antioxidative potency and prevent peroxidative hemolysis of sheep, rat, and rabbit erythrocytes (15). Quercetin and silybin (19) were reported to exert a protective effect by the decrease in the xanthine dehydrogenase/oxygenase ratio observed during ischemia/reperfusion in the rat. The enzyme (xanthine oxidase) implicated in tissue oxidative injury after ischemia/reperfusion is a source of ROS that is formed from a dehydrogenase during ischemia. The protective effect of quercetin and silybin on the xanthine dehydrogenase/oxidase ratio is due to inhibition of the dehydrogenase.

PROOXIDANTS

A prooxidant is an agent that can induce oxidative stress, which is defined as a shift in the prooxidant–antioxidant balance toward oxidant activity. Oxidative stress induced by a prooxidant in a biological system manifests itself as increased production of bioactive free radical species, a decrease or modulation of antioxidant defenses, and/or an increase in oxidative damage. The fine balance between the oxygen center radicals and antioxidants may be dependent on the concentration of prooxidant, oxygen tension, and interactions with other antioxidants.

Vitamins

Carotenoids

Carotenoids are a well-characterized class of pigments widely distributed in nature and responsible for the bright colors of various fruits and vegetables. Some of the more than 600 different carotenoids are well known, such as β-carotene, which is widely used as a precursor of vitamin A, a food colorant, and a food additive (22). These

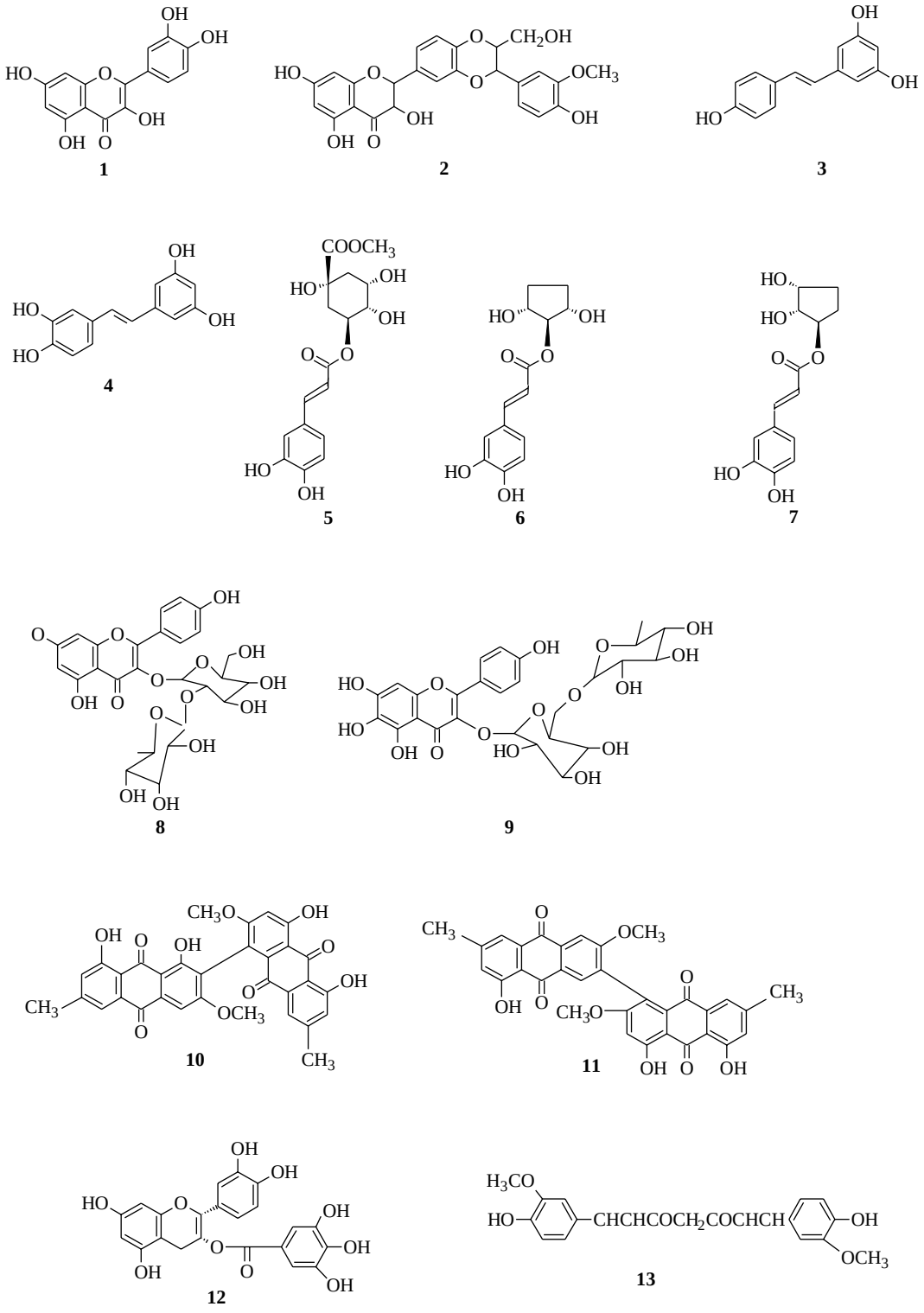


Fig. 2 Phenolic antioxidants isolated from plants; **1**. quercetin; **2**. silymarin; **3**. *trans*-resveratrol; **4**. piceatanmol; **5**. chlorogenic acid methyl ester (16, 17); **6**. 1-(3',4'-Dihydroxycinnamoyl)-cyclopenta-2,5-Diol (16); **7**. 1-(3',4'-Dihydroxycinnamoyl)-cyclopenta-2,3-Diol (16); **8**. kaempferol-3-*O*-neohespheridoside (17); **9**. 5,6,7,4'-Tetrahydroxyflavonol-3-*O*-rutinoside (22); **10**. floribundones I; **11**. floribundones II, **12**. (-)-epicatechin-3-*O*-gallate; **13**. curcumine.

compounds have been shown to function as enhancers of gap–junction communication, stimulants of immune responses, and quenchers of electronically excited species, such as singlet oxygen and triplet sensitizers (23–25). The action of β-carotene and other carotenoids as antioxidants has recently attracted widespread attention. Carotenoids are thought to scavenge free radicals, and the antioxidant action of β-carotene and other carotenoids has been observed with in vitro andin vivo systems (26, 27). However, carotenoids do not have structural features commonly associated with chain-breaking antioxidants. The extensive system of conjugated double bonds in their molecules imparts a prooxidant character and makes them very susceptible to attack by free radical species (Fig. 3).

The development of either a harmful or beneficial cellular response by carotenoids will depend on their antioxidant or prooxidant characteristics, which are determined by various factors in the intra- and extracellular environments such as oxygen tension and β-carotene concentration. When an inappropriate prooxidant activity of carotenoids develops in normal cells, the reactive oxygen metabolites generated could induce damage to lipids, proteins, and DNA. This effect alters normal regulatory functions and can damage cellular integrity or induce neoplastic transformation. Some human intervention trials indicate that carotenoid supplements are of little or no value in preventing chronic disease, such as cardiovascular disease and cancer, and may actually increase lung cancer incidence in smokers (22). In contrast, when carotenoids act as prooxidants in already transformed cells, they could induce beneficial effects, such as inhibiting the growth and development of malignant lesions and/or producing tumor cytotoxic effects (28).

Although it has been reported that carotenoids may prevent normal cells from becoming transformed through their antioxidant activity, there is much evidence indicating that they may also block the growth of cells already transformed through their prooxidant action. Carotenoid autoxidation has been suggested to occur at a higher level in tumor cells than in normal cells. In tumor cells, therefore, it can be hypothesized that the prooxidant properties of carotenoids prevail over the antioxidant properties (29). In addition, some studies have shown that carotenoids act as prooxidant agents selectively in tumor cells by increasing the expression of heat-shock proteins (30) and enhancing the formation of lipid peroxidation products, further reducing the levels of oxygen-protective enzymes and stimulating the expression of tumor necrosis α-factor.

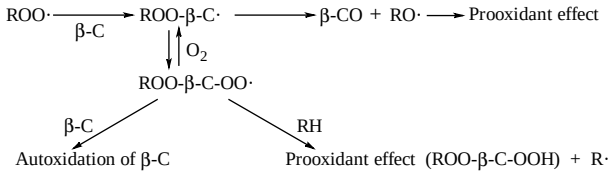


Fig. 3 Proposed reaction pathway of β-Carotene prooxidant activity. β-C, β-carotene; ROO·, peroxyl radical; ROO-β-C·, β-carotene radical; ROO-β-C-OO·, β-carotene peroxy radical; β-CO, β-carotene epoxide; RO·, alkoxy radical. (Adapted in part from Ref. 28.)

Ascorbates

Ascorbate is an essential vitamin that must be taken from external sources. It is marketed as a dietary supplement because of its antioxidant properties. Several epidemiologic studies suggest that antioxidant vitamins in sufficient concentrations inhibit heart disease and cancer. However, there is considerable uncertainty about the optimal level of intake. Substantial evidence shows that ascorbate can also act as an oxidant, depending on the environment in which the molecule is present. It can induce cell death, nuclear fragmentation, and internucleosomal DNA cleavage in human myelogenous leukemic cell lines (29). More recently, it was reported that dietary supplementation of 500 mg/day of vitamin C to healthy volunteers for 6 weeks results in significant prooxidant effects. This is exemplified by an increase in lymphocytes with typical markers of DNA damage, mediated by oxygen radicals such as 8-oxoguanine and 8-oxoadenine (31).

Vitamin C supplementation is able to significantly affect CYP2E1-catalyzed drug metabolism in the rat and is linked to an overgeneration of the superoxide anion in hepatic microsomes. Generation of a superoxide anion induced by vitamin C supplementation provides a plausible explanation for the observed damage to DNA in peripheral blood lymphocytes (32). Ascorbate is a reducing agent in brain tissue homogenate but has an oxidizing effect in brain slices. A hypothesis put forth to explain the oxidative effects of ascorbate in cortical slices proposes that extracellular ascorbate is oxidized to dehydroascorbate which is rapidly carried into cells via a glucose transporter. The dehydroascorbate in cytosol is then reduced back to ascorbate, and, during the reduction process, cellular components are oxidized (Fig. 4) (33).

Tocopherols

Vitamin E, the major lipophilic antioxidant of exogenous origin in tissues, is the collective name for the eight major naturally occurring molecules, four tocopherols and four tocotrienols, that qualitatively exhibit the biological

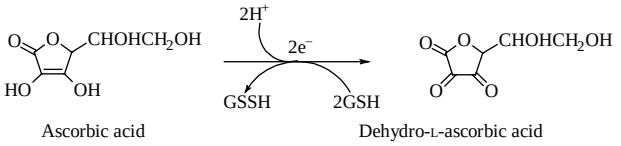


Fig. 4 Structure of the reduced and oxidized forms of vitamin C.

activity of α-tocopherol. α-Tocopherol is generally regarded as the most important lipid-soluble antioxidant in plasma, circulating lipoproteins (34), and tissues, whereas γ-tocopherol and tocotrienols are present in these tissues at much lower concentrations (Fig. 5).

In an investigation of the antitumor activities of α-tocopherol acid succinate and acetate, the acid succinate form inhibited the growth of oral carcinoma cells, while stimulating the growth and differentiation of normal keratinocytes (35). The acetate form increased thymidine incorporation and mutant p53 expression in cancer cells, thereby increasing proliferation and expression of the cyclin regulator p34cdc. Vitamin E treatment reduced the time required for wound healing in the oral cavity and increased the in vitro growth of endothelial cells (36). Platelet adhesion was also inhibited, resulting in a possible reduction in thrombosis. The mechanism of action for this response appears to be blockage of the prostaglandin pathway at the site of cyclooxygenase activity, and it is a plausible mechanism that supports the use of vitamin E in periodontal disease (29).

The antioxidative activity of vitamin E is converted to prooxidant activity when mild conditions were used to initiate oxidation. A prooxidant effect of α-tocopherol on lipid peroxidation was found only when the samples were virtually free of ascorbate, or if the final concentration of ascorbate in the samples was physiologically low. Adding ascorbate to a near-physiological final concentration restored the antioxidant activity of α-tocopherol under mild oxidative conditions (37).

Retinoids

Retinoids, metabolic and synthetic derivatives of vitamin A, have been shown to function as effective antioxidants and inhibit the peroxidation of PUFA in lipid bilayers. For example, several retinoids inhibit ascorbate-dependent, iron-catalyzed lipid peroxidation in rat liver microsomes and brain mitochondria. Retinol palmitate was shown to function as an antioxidant in rat heart and brain tissues (38). However, retinoic acid was shown to stimulate the rate of 2,2′-Azo*bis*(2-Amidinopropane)-initiated autoxidation of linoleic acid in sodium dodecyl sulfate micelles, and this observation may account for the prooxidant effect of retinoic acid in this system (39). In addition, possible detrimental effects of retinoids (e.g. promotion of tumor growth), increase in low-density lipoproteins (LDL) and triglyceride, and exacerbation of preexisting autoimmune disease have all been reported (40).

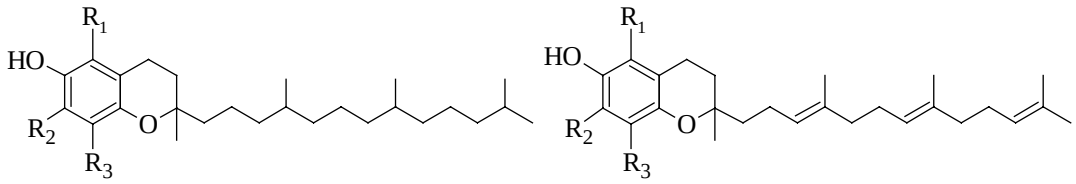
Phenolics

Phenolics are one of the major groups of nonessential dietary components that have been associated with the inhibition of atherosclerosis and cancer. The bioactivity of phenolics may be related to their antioxidant behavior, which is attributed to their ability to chelate metals, inhibit lipoxygenase, and scavenge free radicals. However, phenolics can also function as prooxidants by chelating metals in a manner that maintains or increases their catalytic activity (Fig. 6). Also, polyphenolics reduce metals, thereby increasing their ability to form free radicals from peroxide.

Flavonoids

Flavonoids, especially, those with catechol or phyrogallol groups, obviously are prone to autoxidation reactions (40). In a recent paper by Morgan et al. (41), the prooxidative and antioxidative properties of phenolics from soybeans and other legumes were documented. The primary phenolics (phenolic acids) and flavonoids were able to reduce ferric to ferrous ions and were able to chelate and alter the catalytic activity of iron. Most of the phenolics tested were also able to inhibit oxidation of linoleic acid micelles and ferrous ion-catalyzed oxidation of glutamine synthase, presumably through free radical scavenging and removal of iron from catalytic sites via chelation. Although flavonoids inhibited oxidation in certain systems, they did not protect against all forms of oxidative damage. The phenolics chelated iron, but this metal ion was still catalytically active and able to oxidize both deoxyribose and DNA. Prooxidant activity of phenolics has also been observed for carnosol, carnosic acid, quercetin, rutin, and luteolin (41).

It was also found that pH was essential in determining the oxidative role of phenolics. In general, a decrease in pH increased iron-reducing activity and reduced the ability of phenolics to chelate and inhibit the catalytic activity of iron. Increasing pH increased deoxyribose and DNA oxidation. Inhibition of lipid oxidation was also influenced by pH, with γ-resorcyclic acid being antioxidative at pH 5.8 and prooxidative at pH 7.4. Hydrobenzoic acid was antioxidative, and apigenin-7-glucoside was prooxidative at pH 7.4, yet neither had an effect on lipid oxidation at pH 5.8. These results suggest that the pH of biological tissues could also influence the antioxidative/prooxidative activity of phenolics (42).



	R_1	R_2	R_3		R_1	R_2	R_3
α-Tocopherol	CH_3	CH_3	CH_3	α-Tocotrienol	CH_3	CH_3	CH_3
β-Tocopherol	CH_3	H	CH_3	β-Tocotrienol	CH_3	H	CH_3
δ-Tocopherol	H	CH_3	CH_3	δ-Tocotrienol	H	CH_3	CH_3
γ-Tocopherol	H	H	CH_3	γ-Tocotrienol	H	H	CH_3
Tocol	H	H	H	Tocotrienol	H	H	H

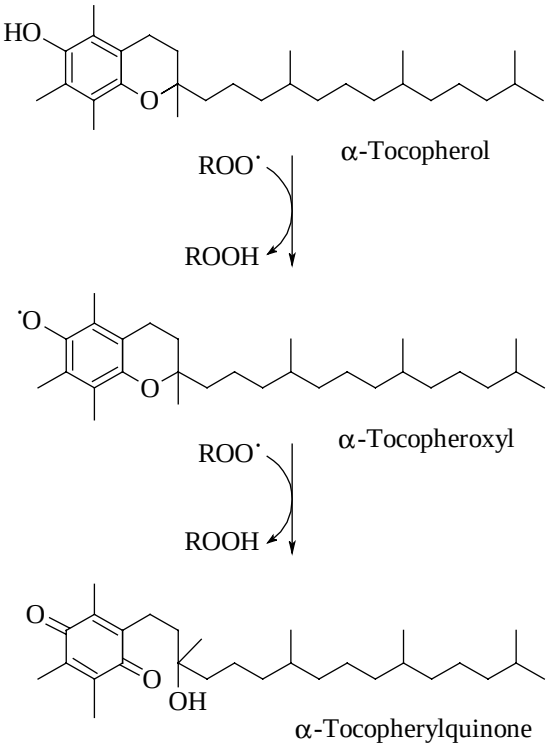


Fig. 5 Chemical structures of the vitamin E groups and metabolic pathway of α-Tocopherol.

A possible mechanism of cytotoxicity in polyphenols may be related to their prooxidant properties (43). Flavonoids autoxidize in an aqueous medium and may form highly reactive HO· radicals in the presence of transition metals. In addition, polyphenols and flavonoids may act as substrates for peroxidase and other metalloenzymes, yielding quinone- or quinomethide-type prooxidant and/or alkylating products. The prooxidant character

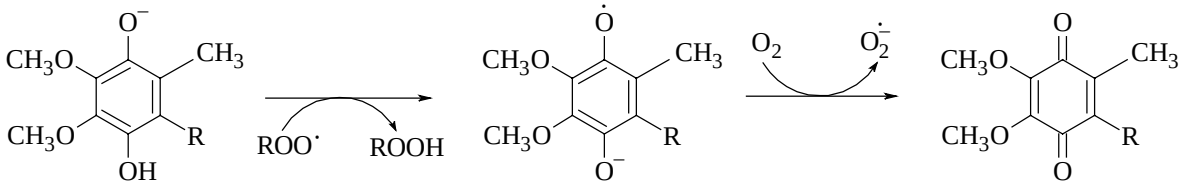


Fig. 6 Antioxidant and prooxidant functions of ubiquinone.

of polyphenol cytotoxicity is supported by the formation of activated oxygen species during gallic acid induced apoptosis, and by the enhancement of gallic and caffeic acid induced apoptosis by non toxic concentrations of copper ions (44).

Catechins, such as (−)-epicatechin and (−)-epigallocatechin abundant in green tea, possess the antioxidative and prooxidative characteristics of Cu^{2+}-induced LDL oxidation. In the initiation phase, LDL oxidation was inhibited by addition of catechin. In contrast, during the propagation phase of LDL oxidation, catechins served as accelerators of oxidation. Depending on redox status, they might form reactive oxidation products such as semiquinones and quinones and function to stimulate oxidative reactions (44).

Quercetin, a highly studied antioxidant flavonoid has the potential to inhibit free radical processes in cells by a) scavenging $O_2^{\cdot -}$, b) blocking lipid peroxidation, c) reacting with peroxyl or lipid peroxyl radicals, d) inhibiting formation of HO·, and e) chelating iron ions. The biological effects of quercetin are believed to result from its antioxidant properties. Recently, it was clearly demonstrated that quercetin could function both as an antioxidant and a prooxidant, depending on concentration and free radical sources and their location in the cell. Also, quercetin was observed to be cytotoxic in a dose-dependent manner. Although the exact mechanism of cytotoxicity has not yet been fully elucidated, it may involve formation of $O_2^{\cdot -}$ or its metabolite *o*-quinone (Fig. 7). Such species are known to be toxic and to bind irreversibly to various cell constituents by covalent binding with sulfhydryl groups or other essential groups (45).

Catecholestrogens

The antioxidant properties of estrogens have been demonstrated in many in vitro and in vivo studies (46–48). For instance, estrogens inhibit the oxidation of LDL, the peroxidation of lipids, and the oxidation of cholesterol. The administration of 17β-estradiol to ovariectomized pigs inhibits the oxidation of LDL. This

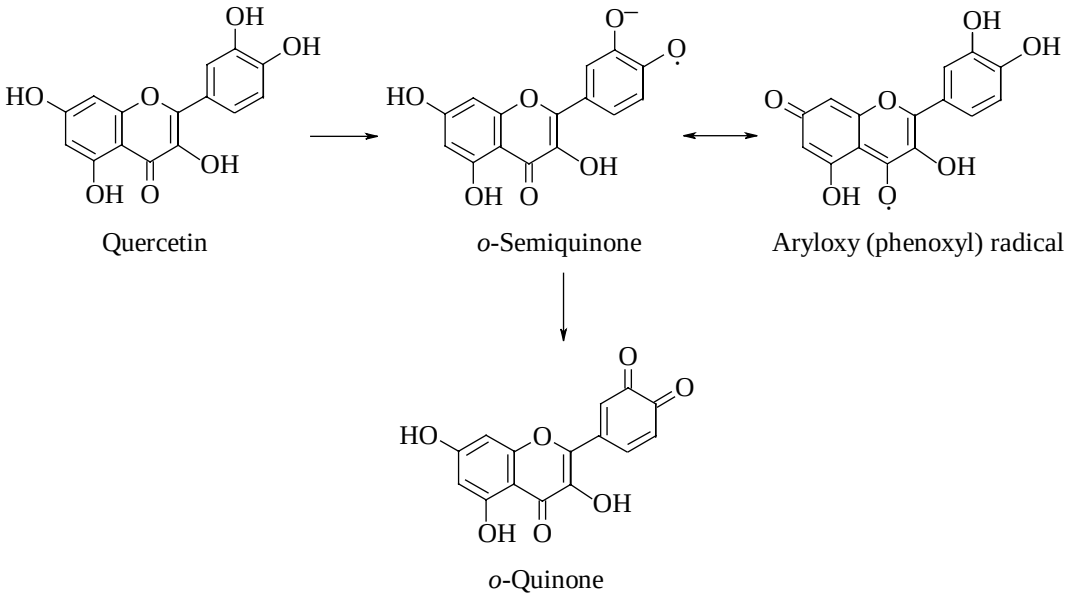


Fig. 7 A simple scheme of quercetin oxidoreductive activation.

effect has also been observed in postmenopausal women after administration of 17β-estradiol. These antioxidant activities of estrogens are believed to explain the lower rate of heart disease in premenopausal women, or postmenopausal women treated with estrogen, compared to that of men (49).

In contrast, prooxidant effects of estrogens have been established in other model systems (50). 17β-estradiol or other estrogens induce single-strand breaks or 8-hydroxylation of guanine bases of DNA in Syrian hamsters treated with these hormones. Moreover, metabolites of estrogen or diethylstilbestrol in the presence of peroxidase and DNA induce 8-hydroxylation of guanine bases. In addition to oxidant-induced damage of DNA, estrogens have also been shown to generate lipid peroxidation and oxygen-radical-mediated oxidation of amino acid residues of proteins, resulting in carbonyl-containing moieties (50).

Estrogens may exhibit either pro- or antioxidant activities depending on the nature of their metabolites and concentrations (Fig. 8). Pharmacological concentrations of parent hormones or their metabolites clearly have antioxidant properties. This inhibitory mechanism may be based on the free radical scavenging action of the phenol moiety of estrogen. In contrast, 2- or 4-hydroxyestradiol enhances the oxidation of LDL by decreasing lag times of lipid peroxidation by 40–50% compared to control values in the absence of estrogen. The catechol structure of catecholestrogens appears to be necessary for this prooxidant activity, as the parent hormones and other estrogen metabolites do not possess any detectable oxidant activity. Therefore, the in vivo prooxidant activity of estrogens may be dependent on their conversion to catecholestrogen metabolites in a specific organ or species. The mechanism of prooxidant activity of catecholestrogens is likely based on the reduction of metal ions, specifically Cu^{2+} to Cu^{+}, by the catecholestrogen metabolites. The lipid oxidation of LDL by Cu^{+} generated hydroxyl radicals, which further initiate oxidation of lipids (51).

DISEASES ASSOCIATED WITH FREE RADICALS

Ischemia/Reperfusion Injury

The syndrome of ischemia/reperfusion (I/R) injury has been characterized in recent years for the heart, brain, intestine, kidney, and other organs. This phenomenon

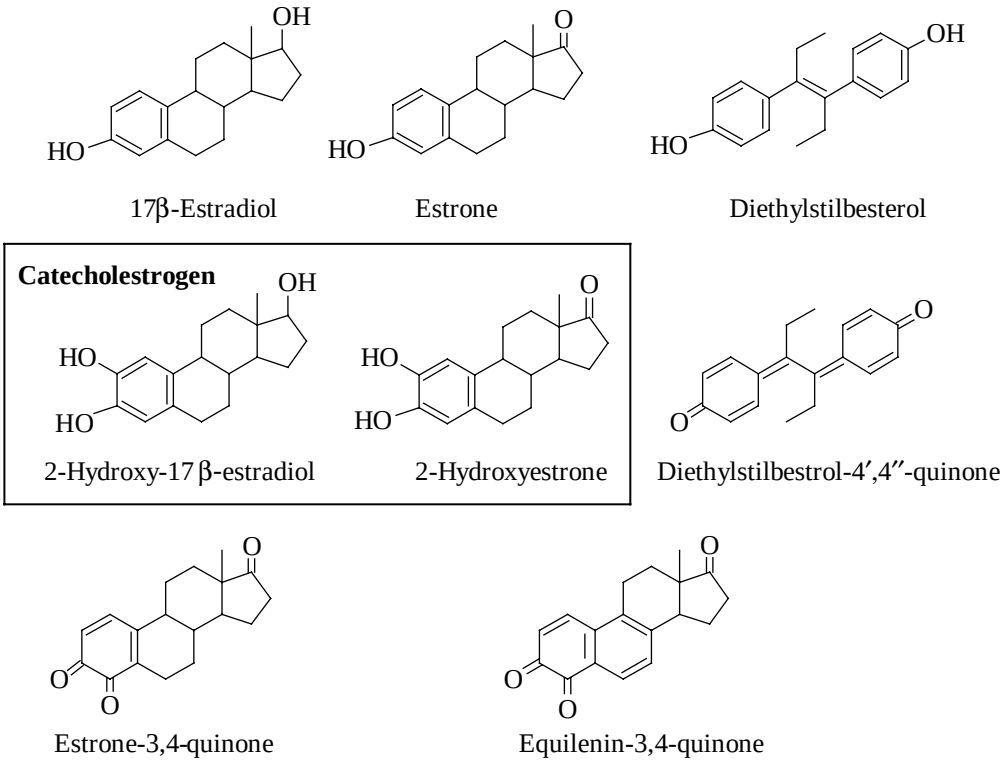


Fig. 8 The metabolites of estrogen.

consists of a paradoxical increase in tissue injury during the reperfusion period in an organ that has sustained relatively minor damage during a period of ischemia. It is now evident that reperfusion tissue injury is mediated through oxidant mechanisms associated with the generation of oxygen-based radicals. ROS have been implicated in both the myocardial dysfunctions that are observed during reperfusion following short periods of ischemia (the stunned myocarium) and the irreversible injury to cardiac myocytes that occurs during reperfusion after longer periods of ischemia (52).

Infusions of high concentrations of the catecholamines, epinephrine, or norepinephrine into experimental animals are known to produce myocellular mitochondrial swelling, myofibrillar disruption, plasma membrane blebbing, and myocardial necrosis. It has been suggested that these cardiotoxic effects result not from the catecholamines themselves but from the production of $O_2^{\cdot -}$ and H_2O_2 formed by a complicated series of reactions during the autoxidation of catecholamines. It was observed that vitamin E deficient rats were more sensitive to the cardiotoxic effects of isoproterenol, whereas myocardial damage induced by this synthetic catecholamine was reduced when the diet was supplemented with vitamin E. However, the results of studies demonstrating protection by antioxidants against catecholamine-induced myocardial necrosis must be interpreted with caution since accumulation of neutrophils, a major source of oxygen radicals, has been observed in such model (53).

Carvedilol, a potent antioxidant, prevents the lipoperoxidation of mitochondrial membranes, which suggests a strong contribution to the known cardioprotective activity of this compound through protection of mitochondrial function (54).

A similar cardioprotective benefit is achieved by agents and antioxidant enzymes that scavenge hydroxyl radicals (or reduce their formation), but not by agents that reduce superoxide anion production. Some compounds of plant origin that have been shown to protect against ischemic injury are procyanidine from *Vitis vinifera* (55), resveratrol from red wine (56), and ginseng extract (57).

Cancer-Carcinogenesis

The progression of tumor formation may be slow, often taking 10 years or more. It is important that carcinogenesis can be viewed as a multistage, microevolutionary process. It is generally agreed that cancer can be derived from a single abnormal cell, and work with experimental systems shows that carcinogenesis is divisible into three major stages: initiation, promotion, and progression. Initiation is a heritable aberration of a cell. Such initiated cells can undergo transformation to malignancy if promotion and progression follow. Initiation appears to be irreversible and can result from DNA damage. Promotion, however, is affected by factors that do not alter DNA sequences; it involves the selection and clonal expansion of initiated cells. This process is partly reversible and accounts for a major portion of the lengthy latent period of carcinogenesis. The final stage of tumor formation is the progression of a benign growth to a malignant neoplasm. There is loss of growth control, an escape from the host defense mechanism, and metastasis.

Certain initiators, such as radiation or chemical carcinogens, can induce the production of various free radicals and subsequent DNA base sequence alteration. In addition, cells of the immune system (e.g., neutrophils and macrophages) produce $O_2^{\cdot -}$ and H_2O_2, which have been associated with the induction of experimental cancers. Oxygen free radicals and methyl radicals are known to damage DNA. In some cases, such free radicals may arise in reactions catalyzed by ferric and cupric ions localized in the vicinity of cellular DNA. Free radical mediated DNA damage can have serious consequences on an organism unless the damage is repaired. Although oxygen free radical effects can lead to DNA damage, they may also directly affect the protein components of the DNA repair apparatus. Unrepaired DNA alterations are inherited as mutations.

Oxygen radicals and related species may also be involved in tumor-promotion. Tumor-promoting phorbol esters not only can induce changes in cellular genes leading to some of the phenotypic characteristics of tumor cells, but they also can stimulate inflammatory leukocytes to release superoxide. The release of superoxide by phagocytic cells following stimulation with phorbol esters is proportional to their tumor-promoting activity. Low levels of both $O_2^{\cdot -}$ and H_2O_2, products of the "respiratory burst," can promote fibroblast growth, possibly fibroblasts that harbor an oncogene or a mutated protooncogene. Also, low levels of superoxide can stimulate growth or growth responses in a variety of cell types when added exogenously to culture medium. In particular, these species stimulate the activation and translocation of protein kinase C, as well as the expression of early growth-regulated genes such as the protooncogenes *c*-fos and *c*-myc. Superoxide and/or hydrogen peroxides might function as mitogenic stimuli through biochemical processes common to natural growth factors. Thus, signaling of growth responses involving released superoxide or hydrogen peroxide may be mediated through the oxidative modification of components of the signal transduction pathway. It is also possible that oxidative

inactivation of serum protein inhibitors allows proteases to remodel the cell surface, thereby facilitating (modulating) the action of normal growth factors (58).

A final and decisive step in carcinogenesis is the invasion and metastatic spread of the tumor to various body spaces and cavities. This appears to be facilitated by the activation of genes for the release of proteolytic enzymes. Although high levels of immune cells appear to favor cell killing, lower numbers of immune cells can favor metastasis. Again, the released superoxide may serve to promote metastatic growth. Alternatively, superoxide could inactivate serum antiproteases, some of which are extremely sensitive to oxidative inactivation (59).

In addition, lipid peroxidation is associated with some phases of carcinogenesis. There is increasing evidence that covalent binding of carcinogens or toxic substances to cellular macromolecules, particularly those carrying genetic information, is the primary event in the initiation of carcinogenesis. Thus, covalent binding to macromolecules could be the basis of many pathological changes induced by toxic substances. The ultimate forms of xenobiotics are believed to be reactive electrophilic metabolites, which combine with nucleophilic groups of macromolecules. It is also possible that miscoding or mutagenesis may be of minor importance in the initial events of chemical carcinogenesis, and that genetic transpositions, including relatively large regions of the genome, may be more relevant (60).

The DNA adducts, deoxyadenosine and deoxyguanosine, which are induced by malondialdehyde, the end-product of lipid peroxidation, accumulate in human breast tissues. These adducts are present at relatively higher concentrations in breast cancer cells compared to normal breast cells (61). In a recent study, serum antioxidative vitamin levels and lipid peroxidation were compared in gastric cancer patients (62). The level of serum ascorbic acid, α-tocopherol, β-carotene, and retinol were assessed. The levels of ascorbic acid in patients with gastric carcinoma were less than one-fifth of that in the control group, and the production of β-carotene and α-tocopherol were decreased, as well.

Neuronal Disease

Lipid peroxidation of biological membranes gives rise to degeneration of synapses and neurons, and may be observed in stroke, or neuronal disorders such as Alzheimer's, Parkinson's and Huntington's diseases. Oxidative stress and damage are accepted features of neural degeneration. The pathological presentation of Alzheimer's disease, the leading cause of senile dementia, involves regionalized neuronal death and accumulation of intraneuronal and extracellular lesions (63). 4-Hydroxynonenal mediates oxidation-induced impairment of glutamate transport and mitochondrial function in synapses (64). Amyloid β-protein may be involved in modulation of membrane lipid peroxidation. Amyloid β-protein fragments 25–35 [A-beta (25–35)] inhibit lipid peroxidation at low concentrations as a result of physicochemical interactions with the membrane lipid layer (65). Further, there is close association between increased levels of the antioxidant enzymes superoxide dismutase and heme oxygenase-1 and cytoskeleton abnormalities found in Alzheimer's disease (66).

DETECTION AND CHARACTERIZATION OF FREE RADICALS

ROS have been involved in the pathogenesis of a variety of human diseases. Their injury potential and pathologic role demand quantitative methods that are diagnostic of the process and that meet basic analytical criteria regarding accuracy, reliability, sensitivity and specificity. In addition, the determination and quantification of antioxidant activity is necessary for the discovery and evaluation of drug candidates (67). However, because of their reactive nature and short half-lives, it is difficult to quantify ROS. Alternatively, analyses involving secondary or end products produced by the attack of ROS on lipids, enzymes, or other cellular components are generally preferred. In spite of recent advances in technology, however, these indirect methods often give misleading results due to their poor specificity and sensitivity.

Chemiluminescence Measurements

Chemiluminescence is the production of light generated from chemical sources. The quantum yield of photons for intrinsic (nonstimulated) or native reactions are low but organic substances are able to undergo an oxidative reaction that can be sufficiently exothermic to produce an emitting state. Generally, the light produced is in the visible range (400–600 nm), but UV or infrared emission is possible.

Due to potential variability and low intensity of native chemiluminescence, enhancer compounds have been introduced (68). These compounds were selected primarily as a result of their high quantum efficiency and photon yield after oxidation. Luminol and lucigenin are enhancers for oxygenation when added to an in vitro biological

system and form high levels of excited-state products and chemiluminescence. These compounds react with all species of oxidants to form 3-aminophthalate and *N*-methylacridone, respectively. The excited electrons in these compounds revert to their ground state with the emission of energy as light, which can be detected by photomultipliers. The sensitivities of luminol and lucigenin vary. Luminol detects H_2O_2, $HO\cdot$, hypochloride, peroxynitrile and lipid peroxy radicals, whereas lucigenin is particularly sensitive to the superoxide radicals. Chemiluminescence may be utilized as a direct noninvasive method for measuring ROS, and for detection of lipid hydroperoxide, phospholipid and cholesterol hydroperoxide if cytochrome *c*–heme is added prior to luminol.

Thiobarbituric Acid (TBA) Assays

The TBA test is perhaps the most widely used method for determining lipid peroxidation. The representative adduct of lipid peroxidation, malondialdehyde, forms a 1:2 adduct with TBA that can be measured by spectroscopy or fluorometry. The general procedure, of which there are numerous variations, simply involves heating a small quantity of the test substance for a defined period of time in an aqueous acidic solution of TBA, and then measuring the absorbance (535 nm) of the red color which is produced in the TBA reaction. It should be considered as an index of oxidative stress that represents primarily lipid peroxidation (69).

Chemical Measurement of Superoxide Radical

The reduction of yellow nitroblue tetrazolium (NBT) to blue formazan is applied as a probe of $O_2^{\cdot -}$ generation in biological systems. This reaction is utilized in demonstrating the role of phagocytes (neutrophils and monocytes/macrophages) in the host response to infection and inflammation. Oxygen is rapidly reduced via a complex NADPH-oxidase system composed of a flavoprotein and a cytochrome. The cytochrome has a sufficiently low midpoint potential to allow the direct catalytic transfer of electrons from NADPH to oxygen resulting in the production of superoxide.

The respiratory burst of phagocytic cells can be assessed by incubating a suspension of cells in an isotonic solution of the yellow oxidized nitroblue tetrazolium dye. During this process, the soluble dye interacts with the cytoplasmic components associating with the oxidant species generated. NBT reduction by activation cells in the presence of superoxide dismutase has been shown to be markedly reduced (70), which suggests the major oxidant species responsible for the reduction of dye to a black-blue deposit called formazan is superoxide. The overall degree of NBT reduction in a given cell population can be quantified by measuring the concentration of reduced NBT or formazan spectrophotometrically.

Cytochrome c Reduction Assay Using a HL-60 Cell Culture System

HL-60 is an acute human premyelocytic leukemia cell line derived by Collins et al. (71), of which about 10% spontaneously differentiate. Various differentiation inducers, such as DMSO, TPA (12-*O*-tetradecanoylphorbol 13-acetate), or retinoic acid, lead to the differentiation of HL-60 cells through the monocyte or granulocyte pathways. HL-60 cells treated with DMSO appear as granulocytes with morphological and functional changes including production of superoxide anion and phagocytosis. Respiratory burst due to phagocytosis produce ROS such superoxide anion in the nonmitochondria oxidase system. In a simple assay procedure, HL-60 cells differentiated by treatment with 1.3% DMSO are stimulated to produce superoxide anion by addition of TPA, and cytochrome *c* oxidized by superoxide anion is measured by absorbance at 550 nm (72).

Xanthine/Xanthine Oxidase Assay

Xanthine oxidase is an obvious candidate for the production of oxygen free radicals. This enzyme is localized in the liver, small intestinal mucosa, and vascular endothelial cells, and catalyzes the hydroxylation of many purine substrates. It converts hypoxanthine to xanthine and then to uric acid in the presence of molecular oxygen to yield superoxide anion. During ischemia, xanthine dehydrogenase is converted to the oxidase form. At the same time, ATP is degraded to hypoxanthine that accumulates in ischemic tissue. On reperfusion, with the readmission of large quantities of molecular oxygen in the presence of high concentrations of hypoxanthine that is the other substrate for xanthine oxidase, there may be a burst of superoxide anion production. With in vitro systems, antioxidant activity can be measured by the absorbance of uric acid (292 nm) which is produced by xanthine oxidase. If a test sample inhibits the enzyme, xanthine oxidase cannot produce uric acid from xanthine (73).

DPPH Assay

DPPH (1,1-diphenyl-2-picryhydrazyl) is a purple-colored stable free radical that is reduced to the yellow-colored

diphenylpicrylhydrazine by free radicals. The DPPH assay measures one electron, such as hydrogen atom donating activity and hence provides a measure of free radical scavenging activity. This assay is suitable for the initial screening of multiple samples, such as plant extracts. Reaction mixtures containing test samples dissolved in DMSO and DPPH in absolute ethanol are incubated at 37°C for 30 min in a 96-well plate and absorbance measured at 515 nm (74).

CONCLUSIONS

As summarized in this article, ROS are involved in reactions of importance in human disease states. A mechanistic understanding of these pathological processes is beginning to emerge. As a result, intervention strategies can be devised and antioxidants are receiving a great attention as potential drugs. While it is clear that prevention of oxidative damage should have a beneficial effect on human health in general, the possibility of prooxidant activity leading to adverse health effects must be strongly borne in mind. A number of assay systems are currently available for thorough characterization of the large number of potential antioxidants which are known, or for the discovery of new chemical entities. With these tools and prudent preclinical and clinical studies, it should be possible to devise dietary strategies or pharmaceutical preparations that will reduce morbidity and mortality.

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